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Identification of a Novel Death Domain-Containing Adaptor Molecule for Ectodysplasin-A Receptor that Is Mutated in *crinkled* Mice

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Summary

Hypohydrotic Ectodermal Dysplasia (HED) is a genetic disease seen in humans and mice. It is characterized by loss of hair, sweat glands, and teeth. The predominant X-linked form results from mutations in ectodysplasin-A (EDA), a TNF-like ligand [1–4]. A phenotypically indistinguishable autosomal form of the disease results from mutations in the receptor for EDA (EDAR) [4, 5]. EDAR is a NF-κB-activating, death domain-containing member of the TNF receptor family [6–8]. *crinkled*, a distinct autosomal form of HED, was discovered in a mouse strain in which both the ligand (EDA) and receptor (EDAR) were wild-type, suggestive of a disruption further downstream in the signaling pathway [9, 10]. Employing a forward genetic approach, we have cloned *crinkled* (CR) and find it to encode a novel death domain-containing adaptor. *crinkled* binds EDAR through a homotypic death domain interaction and mediates engagement of the NF-κB pathway, possibly by recruiting TRAF2 to the receptor-signaling complex. This is an unprecedented example of naturally occurring mutations in ligand, receptor, or adaptor giving rise to the same phenotypic disease characterized by a defect in the proper development of epidermal appendages.

Results and Discussion

EDAR is a NF-κB-activating, death domain-containing member of the TNF receptor family. Unlike many members of the TNF receptor family, EDAR fails to directly bind any of the TRAFS, molecules involved in receptor engagement of the NF-κB pathway (our unpublished data). By analogy to the signaling mechanism of TNFR1 [11, 12], it was quite likely that EDAR, whose intracellular death domain has been shown to be critical in downstream signaling events, might employ a unique death domain (DD)-containing adaptor. Genetic evidence suggests that the gene disrupted in *crinkled* mice might serve as such a candidate [9, 10]. The *crinkled* locus had previously been mapped to a region 6.0 cm to the telomere of mouse chromosome 13 (The Jackson Laboratory). By searching a mouse genomic database, we identified a novel DD-containing exon in the *crinkled* locus. A similar DD-containing exon was also found in the human genome. Using murine embryonic skin and human fetal skin libraries, we cloned both mouse and human cDNAs, respectively, with the predicted open reading frame encoding the newly identified DD. Subsequent genomic and biochemical analyses indicated that disruption of this gene was likely responsible for the *crinkled* phenotype (see below); we therefore tentatively termed the new gene "*crinkled*" (CR).

Sequence analysis reveals that CR contains a DD in its C-terminal region, while its N-terminal region shows no significant homology to any known proteins (Figure 1A). Human and murine CR are highly conserved. A BLAST search [13] revealed that the DD of CR exhibits high homology to murine MyD88 (35% identity, with an E value of 5×10^{-3}). When the sequence of CR was compared to structure-based profiles generated by multiple structure alignment of DD-fold family members using PSI-BLAST [14], statistically significant hits were only found for DD profiles. "Threading" [15] the sequence against a comprehensive fold library comprising 4933 domains, using the program ProFit (ProCeryon Biosciences, Inc.), also strongly indicated that the C-terminal region of CR represents a DD. A multiple sequence alignment between the C-terminal region of CR and known DDs is shown in Figure 1B. Secondary structure prediction using the program PHD [16] suggests helical secondary structure entirely consistent with the illustrated sequence alignment.

PCR analysis of a human cDNA panel showed that human CR (hCR) and human EDAR (hEDAR) have a similar pattern of tissue distribution (Figure 1C), suggesting coexpression of the genes. Interestingly, in situ hybridization analysis revealed that both CR and EDAR are expressed in the basal layer of the epidermis and hair follicles of P2 mice (data not shown), consistent with a role for both genes in hair follicle formation.

To determine the nature of the gene alteration in *crinkled* mice, we designed a PCR strategy based on available mouse genomic sequence to amplify genomic DNA from either *crinkled* or wild-type mice (Figure 2). Searching the human genomic sequence database allowed us to identify the six exons encoding the open reading frame region of CR. We were able to locate the last three mouse exons within a large genomic contig, including the last exon (EX6) that encoded the entire DD of CR (Figure 2). Exon 1 (EX1) and exon 2 (EX2) were found in two distinct genomic fragments that apparently did not assemble into a large contig. Genomic sequence representing mouse exon 3 (EX3) has not yet been found. Regardless, PCR analysis using primers corresponding to individual exons showed that all the exons for mouse CR were deleted in *crinkled* mice. Further mapping of the CR locus indicated that a region ~60 kb upstream and at least 6.2 kb downstream of exon 6 was deleted.

To investigate whether CR was the adaptor molecule involved in EDAR signaling, we first tested for direct interaction between CR and EDAR (Figure 3A). Indeed, the intracellular domain of EDAR bound CR but not

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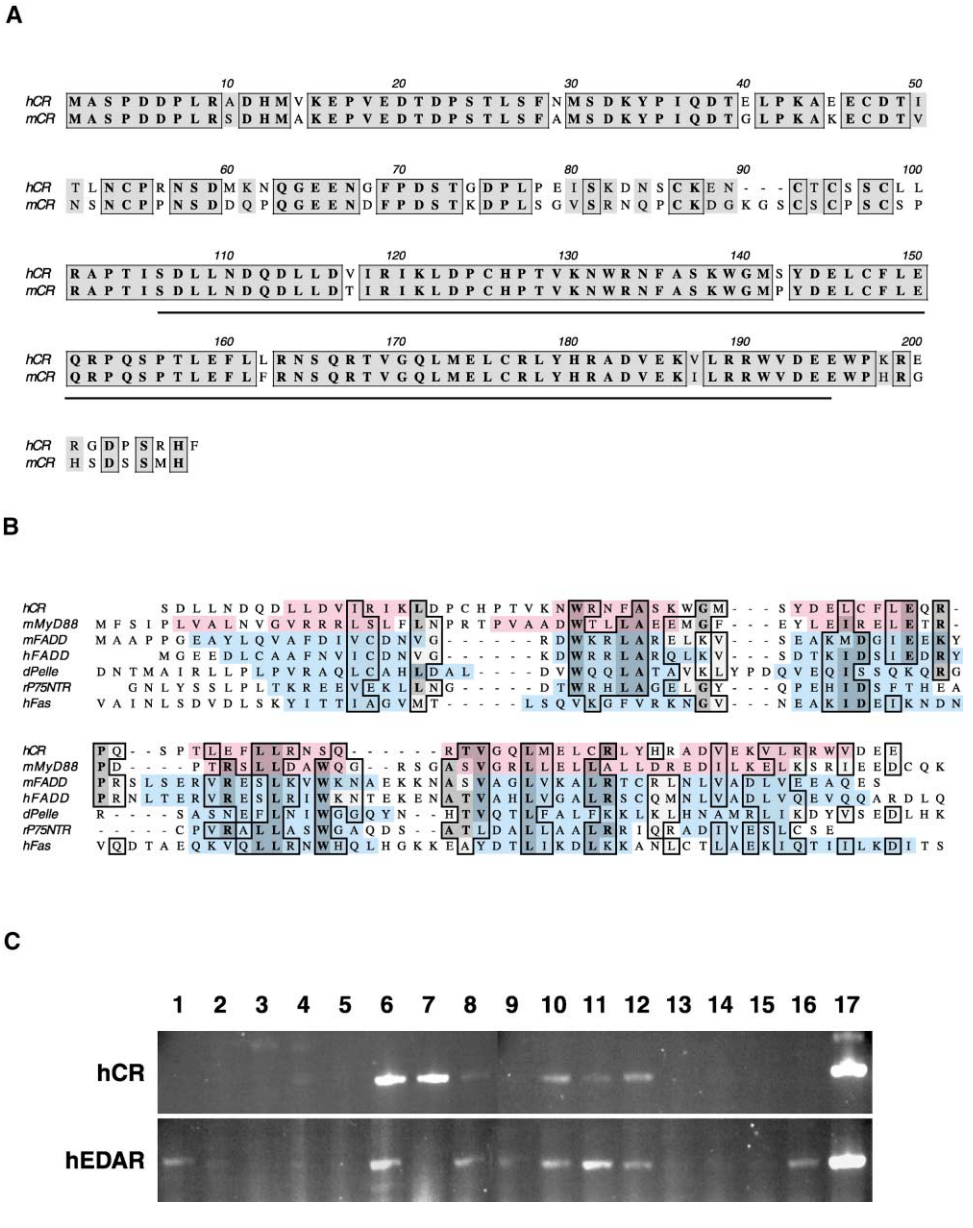

Figure 1. Sequence Alignment and Tissue Distribution of CR

(A) Sequence alignment of human and murine CR. Identical amino acids in human and murine CR are boxed. Conserved amino acids are shaded. The death domain is underlined.

(B) Optimized alignment of the C-terminal DD of CR and selected sequences corresponding to the DDs of murine MyD88, murine FADD, human FADD, *Drosophila* Pelle, rat $p75^{NTR}$, and human Fas. The sequence alignments between the DDs of known 3D structure are based on structural superposition versus mFADD using the program ProSup (ProCeryon Biosciences, Inc.). Those between hCR and the known DDs were optimized with minimal manual intervention, based on sequence-sequence alignments obtained using structure-based profiles with the program PSI-BLAST and sequence-structure alignments obtained using the fold recognition program ProFit (ProCeryon Biosciences, Inc.). Regions of helical secondary structure for the DDs of known 3D structure are indicated in cyan. Regions of predicted helical secondary structure are indicated in pink.

(C) PCR analysis of human multiple-tissue cDNA panel (Clontech). cDNA fragments were amplified using gene-specific primers. Lanes 1 through 16: 1, kidney; 2, liver; 3, skeletal muscle; 4, heart; 5, brain; 6, pancreas; 7, placenta; 8, lung; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16; leukocyte. Lane 17, 10 ng human fetal skin cDNA used as a positive control. hCR, human CR; hEDAR, human EDAR.

RAIDD or TRADD, two other DD-containing adaptors. The binding of EDAR and CR was mediated by a homotypic DD:DD interaction, as an N-terminal truncated construct, CR-DN (amino acids 101–205), that expressed only the DD was sufficient for binding EDAR. Under similar experimental conditions, XEDAR, a related member of the TNFR family, failed to bind CR or CR-DN.

Various TRAF molecules, in particular, TRAFs 2, 5, and 6, have been implicated as intermediaries in receptor-mediated NF-κB activation. Since EDAR is unable to

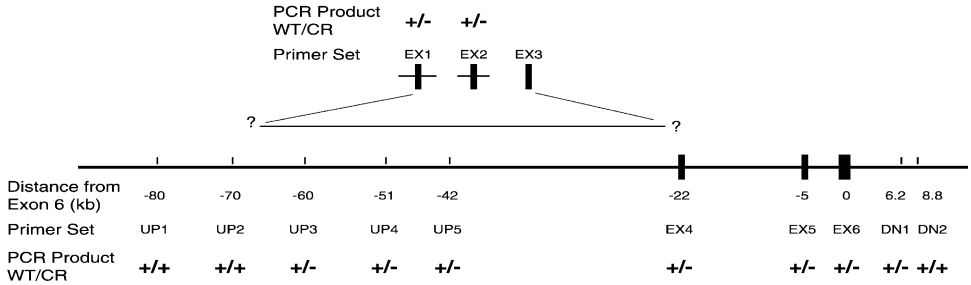

Figure 2. CR Is Deleted in *crinkled* Mice

Primer sets were used as indicated to amplify genomic DNA isolated from *crinkled* mice and control mice (The Jackson Laboratory). Location of the primer sets used in the PCR analysis are indicated by their distance from exon 6 of CR. Six exons corresponding to the open reading frame are shown as black bars. The precise locations of exon 1, exon 2, and exon 3 are not mapped but are predicted to be in a region indicated by the line flanked by question marks. For each primer set, +/+ indicates that an expected PCR product was obtained from both control wild-type DNA and *crinkled* DNA; +/− indicates that an expected PCR product was amplified only from control wild-type DNA but not from *crinkled* DNA.

directly bind TRAFs 2, 5, or 6, (our unpublished data), we decided to examine whether EDAR was able to recruit TRAFs through CR. A strong interaction was observed between CR and TRAF2, but only marginal interactions were seen between CR and TRAF 5 or 6 (data not shown). As shown in Figure 3B, Myc-tagged TRAF2 was readily coprecipitated with Flag-tagged CR. Similarly, Myc-tagged CR was found complexed with Flag-tagged TRAF2. The N-terminal region of CR likely mediated this interaction because CR-DN failed to bind TRAF2 in the same assay. Finally, EDAR, in the presence of CR, was able to recruit TRAF2 (Figure 3C).

Since EDAR is capable of activating NF-κB, overexpression of its adaptor molecule should also activate NF-κB. As expected, exogenously expressed CR was able to activate NF-κB in a dose-dependent manner (Figure 4A). More importantly, CR-DN, which expresses only the DD of CR, was able to serve as a dominant-negative mutant to block EDAR-induced NF-κB activation in 293T cells, which express CR endogenously (data not shown). In contrast, CR-DN had no effect on NF-κB activation by XEDAR (Figure 4B). These data strongly support the notion that CR is a specific adaptor for EDAR in engaging the NF-κB pathway, which is critical for epidermal appendage development [17].

The gene responsible for the X-linked form of Hypohydrotic Ectodermal Dysplasia is ectodysplasin-A (EDA), a TNF-like ligand. An autosomal form of the disorder is

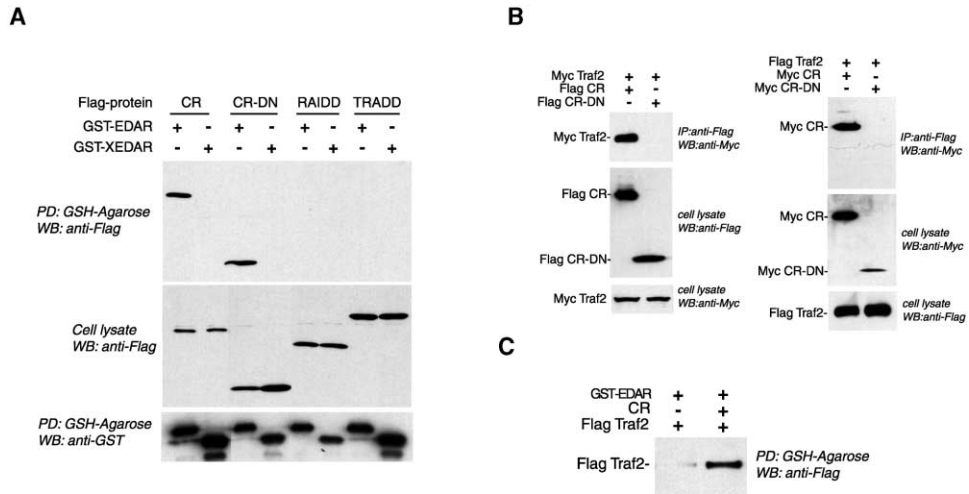

Figure 3. CR Is an EDAR-Specific Adaptor Molecule Capable of Recruiting TRAF2 to EDAR

(A) Interaction between CR and EDAR. 293T cells were cotransfected with plasmid expressing GST fusion protein of either the intracellular region of EDAR (GST-EDAR) or XEDAR (GST-XEDAR) together with Flag-tagged proteins as indicated. GST fusion proteins were precipitated with GSH-agarose beads and the binding of Flag-tagged protein detected by anti-Flag Western blotting (top panel). The expression levels of Flag-tagged proteins (middle panel) and GST-fusion proteins (bottom panel) are also shown. PD, pulldown; WB, Western blotting.

(B) CR binds TRAF2 through the N-terminal non-death domain region. Flag-tagged or Myc-tagged proteins were transiently expressed in 293 cells and cell lysates mixed as indicated. Protein interaction was detected by anti-Flag immunoprecipitation followed by anti-Myc Western blotting. IP, immunoprecipitation.

(C) CR mediates the recruitment of TRAF2 to EDAR. GST-EDAR, Flag-tagged TRAF2, and untagged full-length CR were separately expressed in 293T cells and cell lysates combined as indicated. The protein interaction was determined by precipitation with GSH-agarose beads followed by anti-Flag Western blotting.

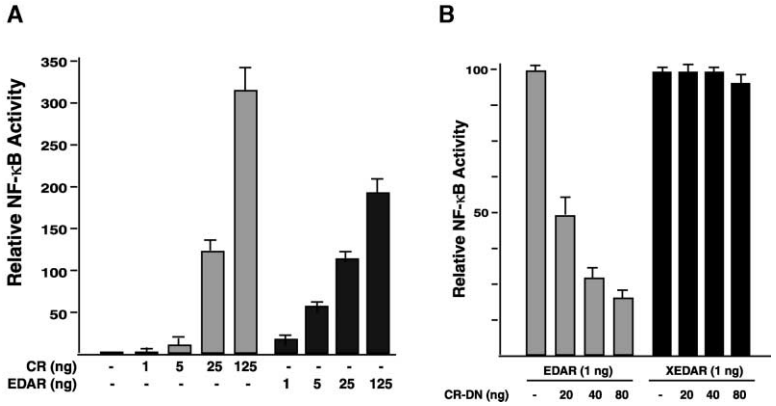

Figure 4. NF-κB Activation by EDAR Is Mediated by CR

(A) Activation of NF-κB by CR and EDAR. 293T cells were transfected with 0.25 μg of ELAM-luciferase reporter gene plasmid, 25 ng pRL-TK, and the indicated amounts of each expression construct. The reporter gene activity was determined with the Dual-Luciferase Reporter Assay System (Promega).

(B) Dominant-negative mutant CR specifically blocks NF-κB activation induced by EDAR. EDAR or XEDAR were transfected alone or together with increased amounts of CR-DN.

due to mutations in EDAR (downless), a death domain-containing member of the TNF receptor family. Not surprisingly, biochemical studies support the genetic evidence that EDA and EDAR function as a receptor-ligand pair. Since *crinkled* mice have a phenotype indistinguishable from tabby (mutation in EDA) and downless (mutation in EDAR) mice, but EDA and EDAR are wild-type, it has long been suspected that *crinkled* may encode a downstream effector of EDAR. In the present report, we have proven this to be the case. CR is a novel DD-containing adaptor whose disruption abrogates EDAR signaling. This gives rise to a phenotype indistinguishable from mutations in other components of this signaling axis that regulates the formation of epidermal appendages.

Experimental Procedures

Molecular Cloning and cDNA Expression Vectors

To obtain the full-length coding sequence of CR, a PCR-based cDNA library screening was undertaken. The murine and human cDNAs containing the entire ORF were generated by PCR using mouse embryonic skin and human fetal skin cDNA libraries. Expression vector pRK5B was engineered using standard recombinant DNA methodology to express various N-terminal epitope-tagged proteins.

PCR Analysis of cDNA Panel

PCR analysis of human cDNA (Clontech) was performed to determine the tissue distribution of human CR and EDAR. For human CR, the primer sequences are forward primer, 5′-TGTACTTGTTC CTCCTGCTTGCTCC-3′; reverse primer, 5′-CATCCGGAGAGGCCAA TGAAGG-3′. For human EDAR, the following primers are used: forward primer, 5′-ACGGAGCTGCCATTTGATTGC-3′; reverse primer, 5′-CAGGATGCAGCATGTGGCTGG-3′.

NF-κB Reporter Assay

293 cells were seeded into 12-well plates and transfected using Fugene 6 reagent (Roche Molecular Chemicals) with 0.25 μg of ELAM-luciferase reporter gene plasmid, 25 ng pRL-TK, and the indicated amounts of each expression construct. Total amount of transfected DNA was kept constant at 1 μg by supplementation with empty vector. Cells were harvested 24 hr after transfection and reporter gene activity determined with the Dual-Luciferase Reporter Assay System (Promega).

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Accession Numbers

The GenBank accession number for human *crinkled* cDNA is AY071862; the accession number for murine *crinkled* cDNA is AY071863.